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HEPATOPROTECTIVE EFFECTS OF L-ORNITHINE-L-ASPARTATE IN TOXIC LIVER INJURY

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Abstract

Aims. L-ornithine-L-aspartate (LOLA) exerts prominent hepatoprotective effects in liver cirrhosis and hepatic encephalopathy. However, its effects on the main pathological syndromes which develop in toxic hepatitis remain elusive. This study is aimed to investigate hepatoprotective action of LOLA in 7 days after toxic liver injury induced by tetrachlormethane (CCl_4) administration in rats.

Methods. Toxic hepatitis was induced in Wistar rats using 50% oil solution of CCl_4 intragastrically (2 g/kg) twice in a 24 hour interval. Intraperitoneal treatment with LOLA (200 mg/kg) was started 6 hours after the second CCl4 administration and maintained for 7 consecutive days. Haematoxylin and eosin staining, biochemical analysis and assessment of oxidant-antioxidant markers were performed. Comparison of multiple groups was performed by One-way ANOVA.

Results. In CCl_4-induced hepatitis, LOLA restores the liver structure and prevents aminotransferases, alkaline phosphatase and gamma-glutamyl transferase elevation. It decreases total bilirubin and total cholesterol concentration. LOLA effectively improves metabolic function of the liver since it impedes CCl_4-induced reduction in serum and liver content of total protein, prevents inhibition in urea synthesis and augments serum and liver content of NO_2^-. LOLA preserves oxidant/antioxidant system in toxic hepatitis by enhancing activity of catalase, manganese superoxide dismutase and copper-zinc superoxide dismutase, increasing reduced glutathione concentration and total antioxidant capacity and decreasing thiobarbituric acid reactive substances level.

Conclusions. In 7 days after CCl_4-induced toxic hepatitis, LOLA prevents cytolysis and cholestasis, improves liver metabolism and protects against oxidative damage.

Keywords: *L-ornithine-L-aspartate, toxic hepatitis, hepatoprotection*

Introduction

L-Ornithine-L-aspartate (LOLA) is a mixture of two endogenous amino acids, L-ornithine and L-aspartate having the well-known benefits for the lowering of blood ammonia and consequently for the treatment of hepatic encephalopathy (HE). The known LOLA effects are to induce hepatic urea synthesis, increase glutamine production in muscles and regulate the relationship between branched aromatic amino acids [1].

LOLA provides key substrates to metabolic pathways involved in the detoxification of ammonia. Ornithine stimulates the activity of carbamoyl phosphate synthetase and aspartate stimulates the activity of arginase by donating nitrogen. Both of these enzymes are necessary for the synthesis of urea. The administration of LOLA decreases the plasma concentration of ammonia and increases the plasma concentration of urea, which proves that LOLA increases the activity of the Krebs cycle [2].

Recent reports suggest that LOLA has, in addition to its established role as an ammonia scavenger, a direct protective effect on the liver per se. It was found that in patients with fatty liver, LOLA attenuates increased blood levels of liver enzymes [3]. LOLA also stabilizes oxidant/antioxidant balance in the liver cells as it provides L-ornithine and L-aspartate as the substrates for glutamate production [4]. The product of LOLA-derived glutamate, namely, glutathione (GSH), is a potent antioxidant that has the requisite properties for the control of oxidative damage [5].

Furthermore, LOLA increases NO synthase (NOS) production with the consequent enhancement in hepatic microperfusion [6] which was confirmed in patients with cirrhosis [7] as well as in an experimental model of chronic liver failure [8].

Although hepatoprotective effects of LOLA in liver cirrhosis are well studied, its effects in toxic hepatitis remain elusive. In acute liver injury, the risk of the patients' death is markedly increased as it is associated with higher blood ammonia levels than in cirrhosis which correlates with increased risk of mortality, more severe encephalopathy, intracranial hypertension, cerebral herniation.

Tetrachlormethane (CCl_4) is a well-known hepatotoxin, which initial metabolite is the trichloromethyl free radical that causes direct liver injury due to cell necrosis caused by altering hepatocyte membrane permeability. CCl_4 induces upregulation of pro-inflammatory mediators, which result in secondary hepatic injury as well. The toxicity of CCl_4 is also associated with free radical formation by the mixed function oxidase system of the liver endoplasmic reticulum [9]. Increased production of oxidants could cause an imbalance in mitochondrial antioxidant defences that may result in a persistent oxidative stress [10].

The aim of our work is to investigate the hepatoprotective effects of LOLA in 7 days after toxic liver injury in rats induced by CCl_4 administration.

Materials and methods

Animals. Adult Wistar strain albino rats were used for the study. Animals were supplied by Central Animal House Facility of Ternopil National Medical University and kept under standard laboratory conditions in polypropylene cages in 12-h light/dark cycle at 25 ± 2°C. Animals were provided with standard diet and water ad libitum.

All the animals received humane care according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985). Experiments performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee on Bioethics of I. Horbachevsky Ternopil National Medical University.

Experimental protocol. The study was performed on 18 white male rats weighing 170-210 g. Animals were randomly divided into 3 groups as follows:

1. Control (C) group (n = 6);
2. CCl4-induced hepatitis (H) group (n = 6);
3. CCl4-induced hepatitis + treatment with LOLA (LOLA) group (n = 6);

To induce acute hepatitis, 50% oil solution of CCl4 was administered intragastrically at a dose of 2 g/kg twice in 24 hour interval. Control animals received the equal amount of saline. Intraperitoneal treatment with LOLA at a dose of 200 mg/kg was started in 6 hours after the second CCl_4 administration and maintained for 7 consecutive days.

Histological study. Histological liver sections were stained with haematoxylin and eosin according to the standard methods.

Biochemical analysis. Serum alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (γ-GT), total bilirubin (TB), alkaline phosphatase (ALP), total protein in serum (TP) and urea were estimated according to the standard protocols using standard kits of reagents "Spaynlab".

Liver concentration of protein was determined according to Lowry et al [11].

Evaluation of nitrite anions content. The content of nitrite anions (NO_2^-) in the liver and serum was determined by a highly specific spectrophotometric method using color reaction with Griess reagent described by Green [12].

Oxidant-antioxidant markers. The copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) activity was measured according to previously described methods [13]. Assessment of catalase activity was performed according to Aebi method [14] and glutathione S-transferase (GST) activity – according to Habig et al [15]. Total antioxidant capacity (TAC) was measured as described before [16]. Reduced GSH was evaluated following Matsumoto et al [17]. Thiobarbituric acid reactive substances (TBARS) was estimated according to the reported procedure [18].

Statistical analysis. Comparison of multiple groups was performed by One-way ANOVA. The data are presented as mean ± SEM. The results were considered statistically significant if the P-value was 0.05 or less.

Results

LOLA improves hepatocyte morphology and inhibits the markers of liver injury in CCl4-induced hepatitis

Figure 1 represents the liver sections from different experimental groups of rats.

In 7 days after toxic liver injury (**H**), induced by CCl4, the lobe structure was considerably altered. The central veins were slightly dilated and did not contain erythrocytes. The trabecular structure was partially preserved, but the sinusoidal lumens were determined only in the presence of macrophages. In the cytoplasm of hepatocytes along the entire lobe, the signs of protein dystrophy were observed, resulting in the increased cytoplasm area and reduced sinusoidal lumens. Slightly hyperchromic nuclei were visualized in most of cells. Intercellular contacts remained partially preserved. Moderate lympho-histiocytic infiltration was observed in the portal tracts.

Treatment with LOLA (**LOLA**) caused the progressive restoration of the liver structure. Central veins were not visualized. The trabecular structure of hepatocytes was predominantly restored, the sinusoidal lumens were determined along the entire size of the lobe, which were expanded more in some areas and contained a small number of erythrocytes and single macrophages. The size of hepatocytes decreased, the cytoplasm became more homogeneous, dystrophic manifestations decreased, the nuclei became normal, and intercellular contacts were restored. The number of binuclear hepatocytes increased. In the portal tracts moderately expressed, mainly focal lympho-histiocytic infiltration was observed.

The major changes in ALT and AST activity are demonstrated in Table 1.

In rats with hepatitis, aminotransferase activity was markedly augmented in 7 days: ALT – by 70,30 %, $p<0.001$, and AST – in 4 times $p<0.001$. LOLA treatment significantly abolished aminotransferase elevation: ALT – by 24,02 %, $p<0.001$, and AST – by 50,86 %, $p<0.001$.

Since LOLA treatment restores the structure of hepatocytes exposed to CCl4 and inhibits aminotransferase elevation, it demonstrates the efficacy in liver cytolysis prevention. This action is partially mediated by NOS-dependent pathway since the use of L-NAME, which is NOS inhibitor, delayed the recovery of hepatocytes and affected the reduction of AST caused by LOLA.

LOLA prevents cholestasis in CCL_4-induced hepatitis

In rats with CCl_4-induced hepatitis, activity of ALP and γ -GT was elevated by 72.63 % and 219.87 %, respectively. In LOLA treated animals, the enzymes' activity was significantly reduced by 21.86 % and 44.95 %, respectively (Table 2).

Total bilirubin concentration increased by 173.55 % in rats with hepatitis. Administration of LOLA caused the reduction of total bilirubin concentration by 40.51 %, $p<0.001$ (Table 2).

A significant increase in total cholesterol concentration by 73.70 % was observed in animals treated with CCl_4. Treatment with LOLA showed the efficacy in total cholesterol concentration reduction (by 24.82 %) (Table 2).

LOLA regulates liver metabolism in rats with acute hepatitis

In rats with CCl_4-induced hepatitis, urea level in the blood was decreased by 33.94 %. LOLA significantly augmented urea concentration by 56.66 % as compared to untreated rats (Table 3).

In the present study, total protein concentration was significantly decreased by 13.50 % in the blood, $p<0.01$, and by 20.33 % in the liver, $p<0.01$, respectively. Treatment with LOLA caused the significant augmentation in total protein concentration both in the blood by 20.27 %, $p<0.001$, and in the liver by 23.49 %, $p<0.01$, as compared to untreated rats (Table 3).

The content of NO_2^- was elevated after CCl_4 administration in the serum by 177.18 %, $p<0.001$. On the contrary, the content of NO_2^- in the liver was significantly decreased by 28.91%, $p<0.001$. LOLA treatment resulted in the decreased production of NO_2^- in the serum by 23.64 %, $p<0.01$, while its synthesis in the liver by increased by 21.99 %, $p<0.01$ (Table 3).

LOLA preserves antioxidant system in CCL_4-induced hepatitis

The activity of catalase in the liver was significantly decreased in group of rats with hepatitis by 15.58 %, $p<0.05$. Administration of LOLA markedly increased catalase activity by 25.40 % (Table 4).

TBARS level, which serves as a marker of lipid peroxidation [19], was increased after CCl_4 administration by 39.68 %, $p<0.01$. LOLA treatment resulted in decreased formation of TBARS by 28.41 %, $p<0.01$ (Table 4).

In rats with hepatitis, the level of reduced GSH decreased by 19.53 %, $p<0.05$, and GST activity was inhibited by 37.1 %, $p<0.001$. LOLA treatment augmented reduced GSH level by 87.43 %, $p<0.001$, as compared to untreated rats (Table 4).

In rats with CCl_4-induced liver injury, activity of both Mn-SOD and Cu, Zn-SOD was reduced under the impact of toxic agent by 16.8 % and 54.72 %, respectively, which was reversed by LOLA administration: Mn-SOD and Cu, Zn-SOD activity elevated by 19.53 % and 56.52 % as compared to untreated rats with hepatitis (Table 4).

Total antioxidant capacity decreased by 12.24 %, $p<0.001$, in rats with toxic liver injury. LOLA treatment markedly elevated TAC by 8.25 % as compared to untreated rats (Table 4).

Discussion

LOLA is a stable salt of natural amino acids ornithine and aspartate which activates the periportal urea cycle in the liver [1], stimulates glutamine synthesis in the liver as well as in the skeletal muscles which leads to the decreased blood ammonia levels [20] and delays the onset of hepatic encephalopathy [8]. However, to date, there is enough data regarding the effects of LOLA in toxic liver injury. In this study we evaluated hepatoprotective effects of LOLA in 7 days after toxic hepatitis induced by CCl_4 administration. We found that LOLA prevents hepatic cytolysis and cholestasis, improves metabolic functions of the liver and exerts significant antioxidant action.

ALT and AST enzymes are of major importance in assessing and monitoring liver cytolysis [21, 22] as their elevation is mainly related to liver cellular damage when hepatocytes undergo necrosis as a result of direct cellular damage or inflammation [21]. Our study shows that the rise in ALT and AST induced by CCl_4 administration is significantly prevented by LOLA treatment. Furthermore, histological analysis demonstrates that LOLA effectively restores the liver structure suggesting its potency against liver cytolysis.

Intrahepatic cholestasis occurs due to leakage of the tight junctions that separate bile canaliculi from blood sinusoids which results in increased concentration of all bile constituents, such as cholesterol, bile acids, and bilirubin [23], and also of enzymes such as ALP and γ-GT [24]. According to the results obtained in our study, LOLA effectively reverses increase in ALP and γ-GT and prevents the elevation in total bilirubin and total cholesterol concentration, thus it could serve as a potent tool in cholestasis prevention.

The liver plays a crucial role in the metabolic processes since it provides synthesis and breakdown of carbohydrates, fats and proteins as well as it is the major site of environmental

compounds detoxification and nutrient metabolism [25]. In this study we demonstrate the alteration in liver metabolism under the toxic influence of CCl_4.

Almost all of serum proteins are synthesized by hepatocytes under physiological conditions. In liver injury, this process might be affected [23]. We found that LOLA impedes CCl_4-induced reduction in serum and liver content of total protein, therefore, it might be potentially used for maintaining liver synthetizing function in acute and chronic hepatic injury.

The significance of hepatic urea production resides in the removal of potentially toxic ammonium ions [1]. Our results show that urea synthesis undergoes the significant inhibition in liver injury which could lead to hyperammonemia and development of HE, characterized by rapid progression of symptoms from discrete changes in mental status to stupor and coma [26]. We found that LOLA prevents inhibition in urea production which corresponds to the other authors' findings [20, 27, 28] suggesting its effectiveness in HE prevention. Mode of ammonia-lowering actions of LOLA is defined by its ability to provide critical substrates for both ureagenesis and glutamine synthesis. Activation of the urea cycle leads to the detoxification of ammonia [1].

The improvement of the liver function could be explained by the following mechanism. After digestion in the gut, LOLA promptly splits into l-ornithine and l-aspartate. The amino nitrogen of the various amino acids may be incorporated into alanine, aspartate, and glutamate via transamination reactions and it may then be reused for the biosynthesis of protein or converted to urea for purposes of excretion [1].

Aspartate may also initiate pyrimidine synthesis and serve as the carbon source for glutamine synthesis. The ornithine serves as an intermediary in the urea cycle. Some ornithine undergoes transamination to form glutamate; some is metabolized by ornithine decarboxylase (ODC) and incorporated into polyamines [1].

The polyamines are the compounds required for cellular differentiation and proliferation. ODC is the first and rate-limiting enzyme in the polyamine biosynthetic pathway. Evidence suggests that ODC and polyamines have an important function in colorectal neoplasia [29] and might be useful to determine tumor aggressiveness in human breast carcinoma [30]. On the other hand, it is well-known that polyamines negatively regulate the translation of ornithine decarboxylase mRNA, thereby controlling their own synthesis. Since LOLA contributes to polyamines production and indirectly affects ODC, it might be involved in cellular differentiation and proliferation. However, to date, there is no study regarding influence of LOLA on polyamines synthesis and ODC activity.

In liver injury, changes in the content of NO_2^- are frequently observed [31]. Since NO_2^- exists as a stable metabolite of nitric oxide, its content could represent NO-synthesizing potency of the body tissues [32]. We have found that the liver production of NO_2^- in toxic hepatitis is reduced while serum NO_2^- is augmented. Decline in liver NO_2^- production could be explained by a profound change in the cellular distribution of eNOS, which leads to its translocation into hepatocyte nuclei. At the same time the high serum nitrite level is likely to be due to the increased concentration of NO, which synthesis is mediated by iNOS. Increased activity of iNOS is the result of proinflammatory cytokines release which is a prominent feature of liver injury [22]. The increased concentration of NO in the flowing blood by the feedback mechanism dramatically inhibits the expression of eNOS. Thus, a relative lack of intrahepatic microcirculation mediator develops, despite there is the overproduction of nitric oxide.

Previously it was established that in patients with cirrhosis [7] and in an experimental model of chronic liver failure [8], LOLA treatment enhanced NOS synthesis. Current work demonstrates that LOLA treatment resulted in the increased production of NO_2^- in the serum and liver. Enhancement of NO synthesis occurs due to the elevated production of l-arginine from l-ornithine via elements of the urea cycle. Since l-arginine is the obligate substrate for NOS, increases in its availability would be expected to result in increased NOS with consequent increases in hepatic microperfusion [1]. NO, produced due to the function of endothelial NOS (eNOS), exerts protective properties in liver injury through the regulation of sinusoidal diameter, prevention of neutrophil adhesion, inhibition of platelet aggregation and adhesion, and scavenging of reactive oxygen species [33].

The oxidative damage caused by free radicals is thought to be a basic mechanism underlying many pathological conditions, including liver injury [24]. The body has a defense mechanism against oxidative stress which consists of some enzymes, some proteins, and a few low molecular weight molecules [34]. In order to investigate antioxidant properties of LOLA, we studied its impact on the major parameters of oxidant/antioxidant system.

SOD enzymes are used as the mitochondrial targets of ROS, and thus their activity might become reduced with ROS exposure [39]. The copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) could effectively eliminate reactive oxygen species (ROS) and maintain the redox balance [35]. Catalase is one more crucial antioxidant enzyme which plays an important role by breaking down hydrogen peroxide and maintaining the cellular redox homeostasis [34]. Catalase deficiency or malfunctioning is associated with the variety of diseases [34]. We have found that exposure to CCl4 induced the failure of potent antioxidant enzymes activity such as catalase, Cu, Zn-SOD and Mn-SOD while LOLA-treatment enhanced their activity in rats with hepatitis as compared to untreated rats which confirms marked antioxidant properties of the drug.

Reduced GSH is the most important intracellular scavengers of free radicals, thereby decreased GSH levels may reflect depletion of the antioxidant reserve [36]. GSH conjugation of the products of membrane lipid peroxidation by GST, the enzyme involved in cellular detoxification, is generally regarded as one of the major cellular defense mechanisms against toxicity [37]. Here we show that reduced GSH concentration is significantly increased after LOLA treatment in rats with hepatitis. Increased production of GSH occurs due to transamination of l-ornithine to glutamate [1].

Assessment of TAC gives an insight into the delicate balance between oxidants and antioxidants [38]. Low TAC could be indicative of oxidative stress or increased susceptibility to oxidative damage [39]. Here we show that the abolishment of TAC, mediated by the toxic influence of CCl_4, was effectively prevented by LOLA administration suggesting its prominent antioxidant action.

Altogether these results indicate that in 7 days after CCl_4-induced hepatitis, LOLA effectively prevents major syndromes which accompany toxic liver injury, such as cytolysis and cholestasis, improves liver metabolism and protects against oxidative stress.

Abbreviations

LOLA, L-ornithine L-aspartate; HE, hepatic encephalopathy; GSH, glutathione; NO, nitric oxide; NOS, nitric oxide synthase; ALT, alanine transaminase, AST, aspartate transaminase, γ-GT, gamma-glutamyl transferase, ALP, alkaline phosphatase, Cu/Zn-SOD, copper/zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; GST, glutathione S-transferase; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; NO_2^-, nitrite anion, ODC, ornithine decarboxylase.

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The authors declsre that there are no conflicts of interest.

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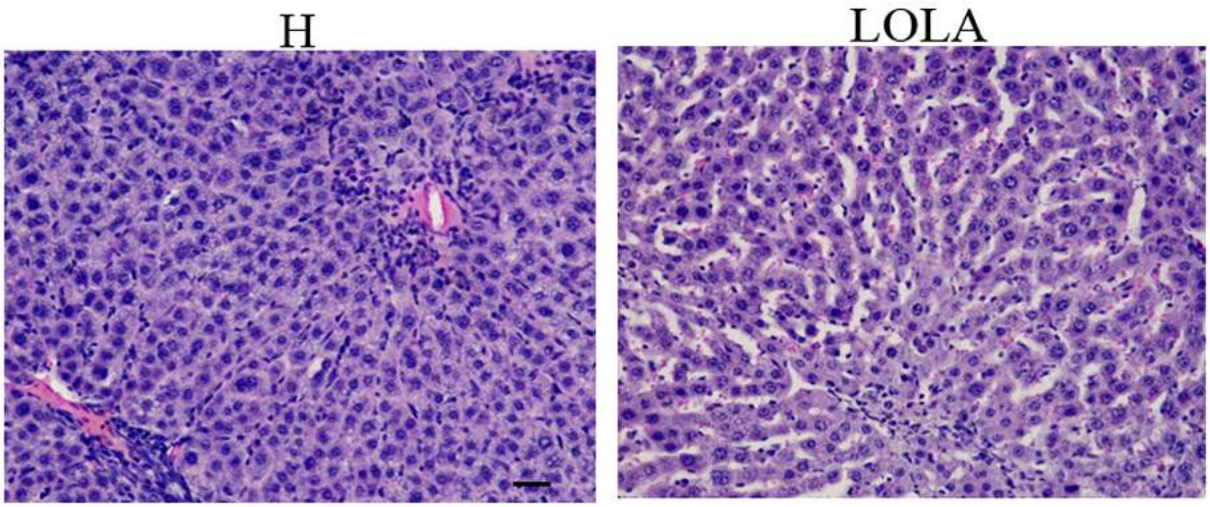

Figure 1. Representative images of rat liver tissues stained with haematoxylin and eosin.
H – CCl_4-induced hepatitis; **LOLA** – CCl_4-induced hepatitis + LOLA treatment Scale bar is 100 µm.

Table 1. ALT and AST activity

	Control	Hepatitis	LOLA
ALT (U/L)	75,75 ± 1,84	129,00 ± 2,14***	98,02 ± 2,73§§§
AST (U/L)	105,35 ± 2,02	417,15 ± 6,12***	205,00 ± 7,05§§§

Values are presented as mean ± SEM.
P-value <0.05 is considered significant.
*** P <0.001 vs control
§§§ P <0.001 vs hepatitis,

Table 2. Parameters of cholestasis

	Control	Hepatitis	LOLA
ALP (U/L)	194.73 ± 5.88	336.17 ± 10.09***	262.68 ± 10.06§§
γ-GT (U/L)	1.03 ± 0.08	3.3 ± 0.16***	1.82 ± 0.19§§
Total bilirubin (mmol/L)	2.50 ± 0.31	6.83 ± 0.18***	4.06 ± 0.34§§§
Total cholesterol (µmol/L)	1.09 ± 0.04	1.89 ± 0.05***	1.42 ± 0.07§§

Values are presented as mean ± SEM.
P-value <0.05 is considered significant.
*** P <0.001 vs control,
§§ P<0.01, §§§ P <0.001 vs hepatitis,

ALP alkaline phosphatase
γ-GT gamma-glutamyl transferase

Table 3. Parameters of liver metabolism

	Control	Hepatitis	LOLA
Urea (mmol/L)	6.36 ± 0,04	4.20 ± 0.13***	6.59 ± 0.25§§
Total protein in blood (g/L)	61.22 ± 0,84	52.95 ± 1,12**	63.68 ± 1.38§§§
Total protein in liver (mg/g)	133.63 ± 2.37	106.46 ± 4.30**	131.46 ± 3.28§§
Serum NO_2^- (µg/l)	1.15 ± 0.05	3.19 ± 0.04***	2.43 ± 0.16§§
Liver NO_2^- (mg/l)	1.89 ± 0.09	1.35 ± 0.03***	1.64 ± 0.05§§

Values are presented as mean ± SEM.
P-value <0.05 is considered significant.
** P<0.01, *** P <0.001 vs control,
§§ P<0.01, §§§ P <0.001 vs hepatitis.

NO_2^- nitrite anion

Table 4. Parameters of oxidant-antioxidant system

	Control	Hepatitis	LOLA
Catalase (µmol/min/mg)	200.33 ± 9.24	169.12 ± 6.60*	212.08 ± 10.60§
TBARS (µmol/kg)	637.65 ± 33.94	890.66 ± 33.81**	674.91 ± 27.64§§
Reduced GSH (µmol/g)	2.64 ± 0.17	2.12 ± 0.11*	3.98 ± 0.23§§§
GST (µmol/min/mg)	1.79 ± 0.05	1.13 ± 0.06***	1.23 ± 0.06^{NS}
Mn-SOD	6.32 ± 0.12	5.26 ± 0.18**	6.28 ± 0.18§§
Cu, Zn-SOD	4.57 ± 0.20	2.07 ± 0.06***	3.24 ± 0.36§
TAC	61.43 ± 0.37	53.92 ± 1.18***	58.37 ± 1.19§

Values are presented as mean ± SEM.
P-value <0.05 is considered significant.
* P<0.05, ** P<0.01, *** P <0.001 vs control,
§ P<0.05, §§ P<0.01, §§§ P<0.001 vs hepatitis,
^{NS} P>0.05 vs hepatitis.